Phosphorylation of DARPP-32 by Cdk5 modulates dopamine signalling in neurons

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The physiological state of the cell is controlled by signal transduction mechanisms which regulate the balance between protein kinase and protein phosphatase activities[1]. Here we report that a single protein can, depending on which particular amino-acid residue is phosphorylated, function either as a kinase or phosphatase inhibitor. DARPP-32 (dopamine and cyclic AMP-regulated phospho-protein, relative molecular mass 32,000) is converted into an inhibitor of protein phosphatase 1 when it is phosphorylated by protein kinase A (PKA) at threonine 34 (refs 2, 3). We find that DARPP-32 is converted into an inhibitor of PKA when phosphorylated at threonine 75 by cyclin-dependent kinase 5 (Cdk5). Cdk5 phosphorylates DARPP-32 *in vitro* and in intact brain cells. Phospho-Thr 75 DARPP-32 inhibits PKA *in vitro* by a competitive mechanism. Decreasing phospho-Thr 75 DARPP-32 in striatal slices, either by a Cdk5-specific inhibitor or by using genetically altered mice, results in increased dopamine-induced phosphorylation of PKA substrates and augmented peak voltage-gated calcium currents. Thus DARPP-32 is a bifunctional signal transduction molecule which, by distinct mechanisms, controls a serine/threonine kinase and a serine/threonine phosphatase.

Numerous first messengers modulate the phosphorylation and dephosphorylation of DARPP-32 at Thr 34 in intact cells, thereby altering activity of protein phosphatase 1 (PP1) and regulating the states of phosphorylation and activities of a variety of downstream physiological effectors[3]. The biological significance of this pathway has been demonstrated using mice containing a targeted deletion of the DARPP-32 gene (DARPP-32$^{-/-}$), which exhibit a markedly altered biochemical, electrophysiological and behavioural phenotype[4].

The amino-acid sequence of DARPP-32 contains consensus phosphorylation sites for proline-directed kinases, including

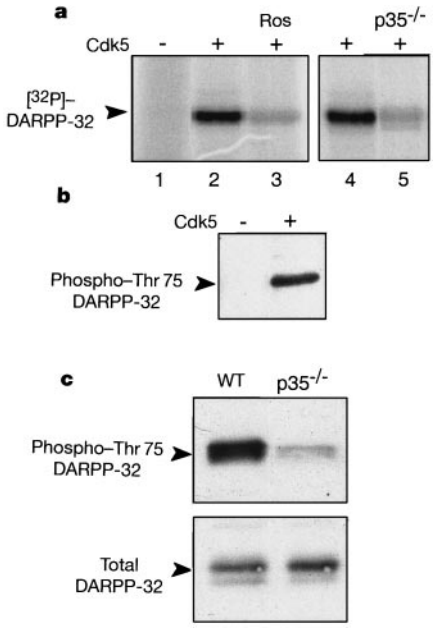

Figure 1 Phosphorylation of DARPP-32 at Thr 75 by Cdk5 *in vitro* and *in vivo*. **a**, Phosphorylation of DARPP-32 by Cdk5 immunoprecipitated from striatum. Roscovitine (Ros) and Cdk5 from p35$^{-/-}$ mice were used as indicated. **b**, Phosphorylation of DARPP-32 at Thr 75 *in vitro* by recombinant Cdk5. **c**, Phosphorylation of DARPP-32 at Thr 75 in intact striatum. Striatal tissue from wild-type (WT) or p35$^{-/-}$ mice were immunoblotted and probed with antibodies against phsopho-Thr 75 (top) and total DARPP-32 (bottom).

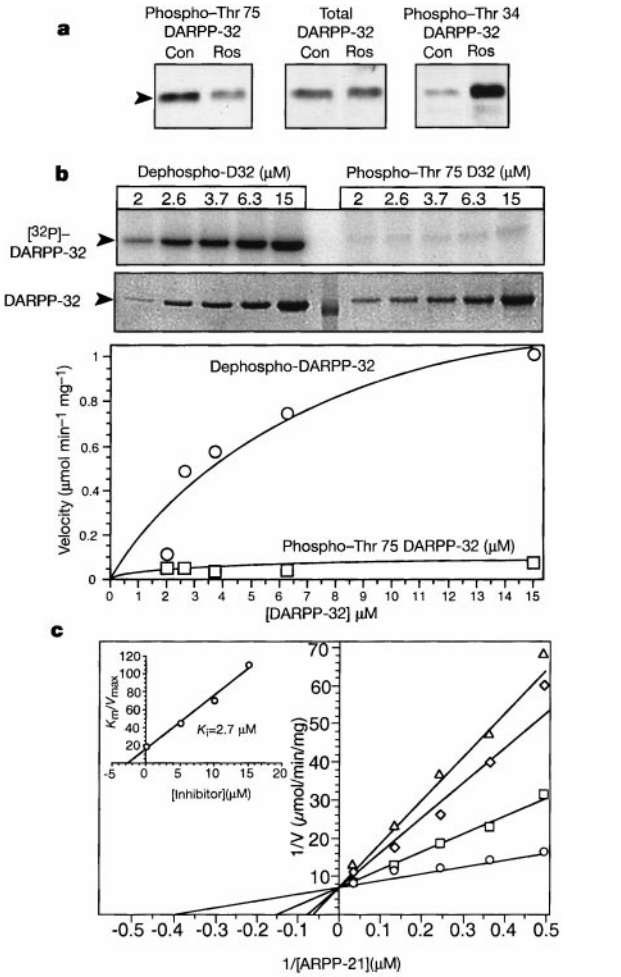

Figure 2 Inhibition of PKA by phospho-Thr 75 DARPP-32. **a**, Effect of roscovitine (Ros) on phospho-Thr 75 and phospho-Thr 34 in mouse striatal slices compared with controls (Con). **b**, Phosphorylation by PKA of dephospho-DARPP-32 (D-32) and phospho-Thr 75 DARPP-32. Top, radiographic image of ^{32}P-labelled DARPP-32. Middle, Coomassie brilliant blue stain. Bottom, quantification of phosphorylation by PKA of dephospho-DARPP-32 and phospho-Thr 75 DARPP-32. **c**, Lineweaver–Burk analysis of PKA phosphorylation of ARPP-21 in the presence of 0 (circles), 5 (squares), 10 (diamonds) or 15 (triangles) μM phospho-Thr 75 DARPP-32. Inset, secondary plot from which the K_i value was derived.

Cdk5, a cyclin-dependent kinase family member which, together with its non-cyclin cofactor p35, is present in post-mitotic neurons expressing high levels of DARPP-32 (refs 5, 6). Cdk5, immunoprecipitated from mouse striatal homogenates, phosphorylated purified DARPP-32 *in vitro* at a linear rate over at least 60 min (Fig. 1a; and data not shown). Phosphorylation was greatly reduced by the addition of the specific Cdk5 inhibitor, roscovitine[7]. DARPP-32 was much less efficiently phosphorylated by Cdk5 immunoprecipitated from striatal homogenates of $p35^{-/-}$ mice (Fig. 1a).

Purified DARPP-32 was phosphorylated *in vitro* by recombinant glutathione *S*-transferase (GST)–Cdk5 (Fig. 1b) and by recombinant Cdk1. Proteolytic digestion, high-performance liquid chromatography purification, phosphopeptide mapping, microsequencing, and MALDI-TOF matrix-assisted laser desorption ionization-time of flight mass spectrometry analysis indicated that the site of phosphorylation by either Cdk5 or Cdk1 was Thr 75. A recombinant protein containing a Thr75Ala mutation was not phosphorylated, indicating that Thr 75 is the only site phosphorylated by Cdk5 and Cdk1. In contrast, mitogen-activated protein kinase, another proline-directed kinase, did not phosphorylate DARPP-32 at a significant rate, and no phosphorylation at Thr 75 could be detected (data not shown).

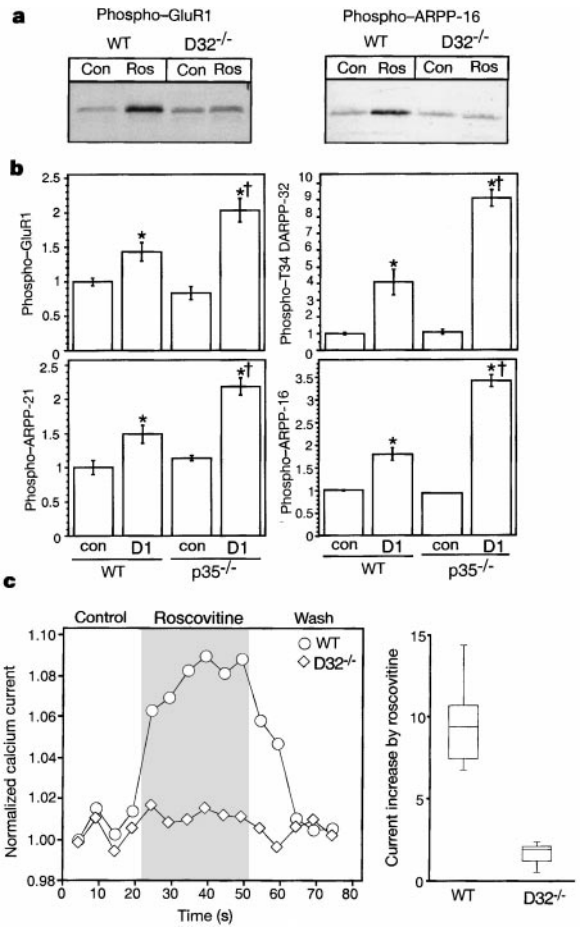

Figure 3 Effect of phospho-Thr 75 DARPP-32 on PKA activity in intact neurons. **a**, Effect of roscovitine on phosphorylation of GluR1 and ARPP-16 in striatal slices from wild-type and DARPP-$32^{-/-}$ mice. **b**, Effect of the selective D1 agonist SKF 81297 (D1) on PKA activity in wild-type and $p35^{-/-}$ mice. Antibodies against the PKA phosphorylation sites of proteins indicated on the left of each panel were used (phospho–GluR1, $n = 4$; phospho-Thr 34 DARPP-32, $n = 6$; phospho-ARPP-21, $n = 4$; phospho-ARPP-16, $n = 4$; asterisk, $P < 0.05$ versus control; cross, $P < 0.05$ versus wild-type D1, by analysis of variance, Newman–Kuel post-hoc test). **c**, Effect of roscovitine on whole-cell Ca^{2+} current in striatal neurons. Left, peak Ca^{2+} current versus time. Right, summary of the roscovitine-induced increase in Ca^{2+} current.

To monitor DARPP-32 phosphorylation by Cdk5 directly *in vitro* and in brain tissue, we generated a phosphorylation state-specific antibody which detected DARPP-32 only in its Thr 75-phosphorylated state (Fig. 1b). DARPP-32 was phosphorylated at Thr 75 in freshly prepared adult mouse striatal tissue (Fig. 1c). Comparison of phospho-Thr 75 and total DARPP-32 standards indicated that the stoichiometry of Thr 75 DARPP-32 phosphorylation was ~0.26 under basal conditions. The level of DARPP-32 in striatal neurons has been estimated to be ~50 μM (ref. 8). Therefore, the basal concentration of phospho-Thr 75 DARPP-32 was ~13 μM. The basal level of phospho-Thr 75 was reduced by 75% in $p35^{-/-}$ mouse striatum, whereas the total level of DARPP-32 was unaffected (Fig. 1c).

The role of DARPP-32 phosphorylated at Thr 75 was assessed in striatal slices from adult mice. Inhibition of Cdk5 by 10 μM roscovitine reduced phospho-Thr 75 DARPP-32 by 73% without affecting the total level of DARPP-32 (Fig. 2a). Remarkably, 10 μM roscovitine also increased phospho-Thr 34 DARPP-32 10-fold from a basal stoichiometry of 0.01 mol mol^{-1}. Dose–response studies indicated that this concentration of roscovitine caused a maximal increase in phospho-Thr 34 DARPP-32 and a maximal decrease in phospho-Thr 75 DARPP-32. We used a biochemical approach to determine the mechanism underlying this reciprocal relationship.

The ability of PKA to phosphorylate Thr 34 of DARPP-32 *in vitro* was virtually abolished by prior phosphorylation at Thr 75 (Fig. 2b). To test whether this effect was due to phospho-Thr 75 acting as an inhibitor of PKA, the ability of the purified catalytic subunit of PKA to phosphorylate several other well-characterized substrates was assessed in the absence and presence of added phospho-Thr 75 DARPP-32. The presence of 15 μM phospho-Thr 75 DARPP-32 markedly reduced the rates of phosphorylation by PKA of inhibitor-1 (a DARPP-32 homologue[2]), ARPP-21 (a PKA substrate enriched in the basal ganglia[9]), synapsin I and histone H1 (data not shown). Kinetic analysis of ARPP-21 phosphorylation in the presence of various concentrations of phospho-Thr 75 DARPP-32 indicated that phospho-Thr 75 DARPP-32 inhibited PKA by a linear competitive single-site mechanism (Fig. 2c). A K_i value of 2.7 μM was derived from a secondary plot of the kinetic data (Fig. 2c, inset).

To determine whether phospho-Thr 75 DARPP-32 inhibits PKA in intact neurons, we assessed phosphorylation of known substrates in mouse striatal slices treated with roscovitine to inhibit Cdk5. Roscovitine increased phosphorylation of the GluR1 subunit of the

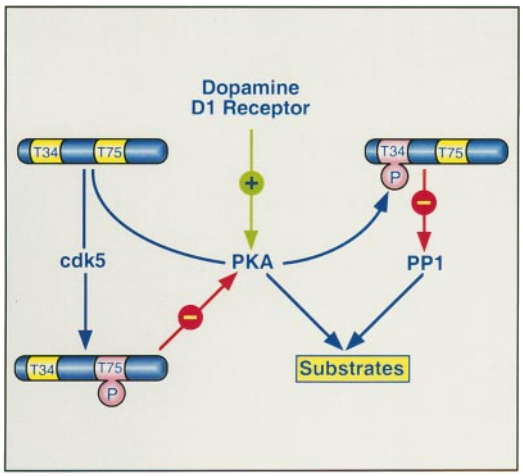

Figure 4 Model illustrating dual effects of phosphorylation of DARPP-32 in regulation of PKA and PP1 activities. Dopamine D1 receptor activation of PKA and phosphorylation of Thr 34 by PKA converts DARPP-32 into an inhibitor of PP1. The activation of PKA and inhibition of PP1 synergistically increase phosphorylation of various substrates[3]. Conversely, phosphorylation of DARPP-32 at Thr 75 by Cdk5 causes inhibition of PKA and activation of PP1, and synergistically reduces phosphorylation of various substrates.

AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor 3.2 ± 0.5-fold ($n = 7$), as assessed using an antibody specific for phospho-Ser 845, a PKA phosphorylation site[10] (Fig. 3a). No roscovitine-induced increase in phospho-Ser 845 GluR1 was observed in striatal slices from adult DARPP-32$^{-/-}$ mice[4], providing strong support for the involvement of phospho-Thr 75 in this action of roscovitine. Total GluR1 levels were unaffected (data not shown). Similar results (2.5 ± 0.2-fold, $n = 2$) for wild-type and DARPP-32$^{-/-}$ mice were observed for phosphorylation of ARPP-16 (a PKA substrate enriched in striatum[11]). These results indicate that regulation by Cdk5 of the phosphorylation of DARPP-32 at Thr 75 has a strong effect on PKA activity in intact cells.

Dopamine achieves many of its effects in neurons by activating the D1 class of dopamine receptors, which are positively coupled to the activation of PKA[3]. To determine whether phospho-Thr 75 DARPP-32 modulates the actions of dopamine in intact cells, the ability of a D1 receptor agonist (SKF 81297) to induce phosphorylation of substrates for PKA was compared in wild-type and p35$^{-/-}$ mice. For this purpose, the state of phosphorylation of GluR1, DARPP-32, ARPP-21 and ARPP-16 was determined using phosphorylation state-specific antibodies. SKF 81297 increased the phosphorylation of each of these substrates in the wild-type mice, and did so to an even greater extent in the p35$^{-/-}$ mice (Fig. 3b). No significant difference was observed between wild-type and p35$^{-/-}$ mice in basal levels of phosphorylation of any of these substrates. These data indicate that Cdk5 phosphorylation of DARPP-32 at Thr 75 can modulate dopamine signalling in the striatum.

We also assessed the physiological consequences of PKA inhibition by phospho-Thr 75 DARPP-32. Voltage-gated Ca^{2+} channels, which are regulated by PKA, are important targets for dopaminergic signalling in the striatum[12–14]. Therefore, we analysed this class of channel using patch-clamp recordings of dissociated striatal neurons (Fig. 3c). Application of roscovitine enhanced whole-cell Ca^{2+} current in wild-type neurons (9.7 ± 1.0%, $n = 7$). This effect was virtually abolished in neurons from DARPP-32 knockout mice (1.6 ± 0.3%, $n = 5$, $P < 0.01$, paired t-test). The dose–response curve for the effect of roscovitine on Ca^{2+} influx was similar to those observed for regulation of phospho-Thr 75 and phospho-Thr 34 of DARPP-32, with a maximal effect at 10 μM (data not shown). These data indicate that phosphorylation of Thr 75 of DARPP-32 may be able to modulate the physiological state of striatal neurons.

Protein kinases and phosphatases are localized to their substrates by targeting and scaffolding molecules[15,16]. In addition, protein kinases and phosphatases associate with each other in signalling modules[17]. Our data show that, through phosphorylation of distinct sites, DARPP-32 can regulate both classes of activity (Fig. 4). This dual action appears to be especially important in regulating the efficacy of dopaminergic neurotransmission. Our results also indicate a novel role for Cdk5 in the actions of dopamine. The ability of one protein to regulate both an important protein kinase and an important protein phosphatase represents a new mechanism by which cell signalling pathways may be integrated. □

Methods

Purified recombinant DARPP-32 was phosphorylated by Cdk5 immunoprecipitated from 100 μg of acutely dissected striatal homogenate as described[18]. Immunoblotting showed that the immunoprecipitated complex from wild-type mice contained both Cdk5 and its cofactor p35 (antibodies from Santa Cruz Biotechnology, data not shown). Reaction mixtures containing 600 μCi ml^{-1} [γ-^{32}P]ATP were incubated for 0–60 min, with 50 μM roscovitine where applicable, followed by addition of gel loading buffer (final concentrations, 1% SDS, 12% glycerol, 25 mM DTT) and boiling for 5 min. Samples were subjected to SDS-PAGE (15% acrylamide), gels were dried, and radiographic images were generated using a PhosphorImager (Molecular Dynamics). DARPP-32 was also phosphorylated by recombinant Cdk5 in reaction mixtures containing 10 μM DARPP-32, 200 μM ATP, 30 mM MOPS (pH 7.2) and 5 mM $MgCl_2$. Samples were incubated for 60 min with or without partially purified GST–Cdk5 and GST–p25 (active fragment of p35) at 30 °C. Reaction mixtures were processed as above, followed by electrophoretic transfer to nitrocellulose and immunoblotting using a phospho-Thr 75 phosphorylation state-specific antibody prepared as described[19].

For kinetic analysis of PKA phosphorylation of dephospho- and phospho–Thr 75 DARPP-32, purified protein was phosphorylated to a stoichiometry of 0.93 mol mol^{-1} using native Cdk1/Cyclin B purified from sea star. Phospho-Thr 75 DARPP-32 was precipitated from the reaction mixture with 5% TCA, resuspended in 1 M Tris-HCl (pH 8.8), and dialysed against 10 mM HEPES (pH 7.4). Dephospho- and phospho-Thr 75 DARPP-32 were phosphorylated by PKA in the presence of [γ-^{32}P]ATP as described[20]. For kinetic analysis of inhibition of PKA phosphorylation of ARPP-21, recombinant ARPP-21 was used and reactions were carried out as described[9]. Phosphorylation was quantified by PhosphorImager analysis.

We prepared striatal slices using standard methodology[21]. Slices from either wild-type or DARPP-32$^{-/-}$ mice were incubated for 1 h in the absence or presence of 10 μM roscovitine. Slices from wild-type and p35$^{-/-}$ mice were incubated for 10 min in the absence or presence of 1 μM SKF 81297. Slices were homogenized by sonication in boiling 1% SDS and 50 mM NaF. Equal amounts of protein were processed as described using 10–20% acrylamide gradient gels and immunoblots were carried out with antibodies against phospho-Ser 845 GluR1 (ref. 10), phospho-Thr 34 DARPP-32 (ref. 22), phospho–Ser 55 ARPP-21 (ref. 23), and phospho-Ser 88 ARPP-16.

For electrophysiology, peak voltage-gated Ca^{2+} current was recorded in acutely dissociated striatal neurons from wild-type and DARPP-32$^{-/-}$ mice in response to bath application of roscovitine. Standard whole-cell patch-clamp techniques and data analysis were used as described[12].

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